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# ATTRACTION, FEEDING, AND REPELLENCY RESPONSES IN MUTANT STRAINS OF *AEDES AEGYPTI*[1]

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ABSTRACT. In a laboratory olfactometer, 12% of female *Aedes aegypti* with a marker gene for red eye (*re*), 0.7% of females with a marker gene for white eye (*we*), and 54.1% of females with normal (*norm*) eye color were attracted to odor from a human hand. When a synthetic attractant blend was used in place of the hand, the attraction rate was 7%, 0.3%, and 35.4%, respectively. On average, *re* females required significantly less time (76.8 sec) than *we* (189.6 sec) or *norm* (176.7 sec) females to locate, land on, and probe human skin but no difference was found between mosquito strains in the time required for females to bloodfeed to repletion on a restrained guinea pig. Differences among mosquito strains in the repellency of 15% diethyltoluamide (deet), 6.65% deet, and 10% citronella were not significant.

**KEY WORDS** Attractant, behavior, mosquito, mutant, repellent

## INTRODUCTION

Not infrequently, the use of insecticides for managing mosquito populations and for controlling the spread of mosquito-borne disease agents, such as West Nile virus, conflicts with other societal priorities. Moreover, resistance to insecticides in mosquitoes, or to parasiticides in the pathogens they transmit, has limited the efficacy of strategies that rely on chemical tools to protect humans and animals from mosquito-borne disease. One increasingly apparent need in this regard is for the development of alternative insect control technologies that can be used to augment or replace insecticides.

This is an attainable goal. In fact, insecticide-free control technologies have been developed for some pest and vector species. Screw worm fly eradication in North and Central America and elimination of *Glossina austeni* from Zanzibar (both using the sterile insect technique [Krafsur 1999]) are examples. And although the feasibility and impact of many biologically based mosquito control methods have yet to be demonstrated, research in this area continues to be driven by the need for alternatives to insecticides. In the case of genetic control, for example, scientists seek to manipulate the mosquito genome to produce an organism incapable of transmitting pathogens (Carlson 1996, Collins and James 1996). One approach to achieving this objective is to modify mosquito feeding and host-seeking behaviors to block the transmission of disease agents to hosts (Scott et al. 2002).

The fitness of genetically modified mosquitoes in nature, compared with their normal-type counterparts, is critical to the success of genetic control efforts. But laboratory and field experiments to characterize this factor can be complicated by an inability to distinguish treatment groups. The use of genetic marker strains (with mutant genes) that manifest a detectable phenotype, such as eye color (Bhalla 1968a) or green fluorescent protein (Chalfie et al. 1994), is one way to surmount the problem. For example, Seawright et al. (1975) used markers for red eye (*re*) color to determine mating competitiveness in an insular population of male *Aedes aegypti* (L.) heterozygous for a translocation. The critically important assumption in such studies is that no difference exists in fitness or performance parameters when wild-type and marker strain or transgenic mosquito populations are compared.

We tested this hypothesis in the study reported here by determining if selected responses in female *Ae. aegypti* females differed among females with wild-type (black) eye color compared with eye color mutant strains. The responses we tested were attraction to a human host and to synthetic attractant in a laboratory olfactometer; the time required to complete host approach, landing, and probing; bloodfeeding time; and the repellency of diethyltoluamide (deet) and citronella.

Data from these comparisons are of fundamental importance for at least 2 reasons. First, under field conditions, differential attraction and feeding responses (between normal and marker strain or transgenic mosquitoes) are a potential source of sampling error that can result in misinterpretation of treatment effects. Second, we do not know if marker strains of mosquitoes attack human hosts and feed (or fail to feed) assortatively in the presence of repellent.

The information from this study will increase our knowledge of host location and feeding responses in mutant mosquito strains and will improve our

[1] Written informed consent was obtained for all human subjects used in this study in accordance with protocol IRB-01 #445-96 as approved by the University of Florida, Health Sciences Center, Institutional Review Board for Human Subjects. The use of animals in this research was reviewed and approved (project number A057) by the University of Florida, Institutional Animal Care and Use Committee, Gainesville, FL.

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understanding of how these organisms respond to repellents compared with wild-type mosquitoes.

## MATERIALS AND METHODS

*Mosquito genetic strains and rearing:* We studied 3 phenotypically distinct forms of *Ae. aegypti*: those with normal (*norm*) wild-type (black) eye color and 2 strains with marker genes, for either red eye (*re*) or white eye (*we*). The *norm Ae. aegypti* were obtained from a laboratory colony maintained at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE) for more than 36 generations. Colonies of the *re* and *we* strains were obtained from the Vector Biology Laboratory, University of Notre Dame, Notre Dame, IN in 1970 (Bhalla 1968a, 1968b; McDonald and Rai 1970) and have been maintained in quarantine at CMAVE since that time (Seawright et al. 1975).

Larvae and adults of each mosquito strain were maintained separately in the same insectary with standard environmental conditions of photoperiod (16:8 h light : dark), temperature (27 ± 1°C), and relative humidity (70–80%). Eggs were obtained from female mosquitoes (in stock cages) provided bovine blood through artificial membranes. All mosquitoes used in this study were 5- to 7-day-old nulliparous females.

We used the method described by Mourya et al. (2002) to verify the purity of each mosquito strain in each generation. To do this, adults were collected 6 h after emergence (each strain was processed separately), anesthetized with $CO_2$, placed on a chill table, and the eye color in each individual was determined at 100× magnification. Mosquitoes exhibiting a mutant phenotype were transferred to the appropriate stock cage and allowed to recover.

*Attraction:* A triple cage olfactometer (Posey et al. 1998) was used to determine the responses of *re, we,* and *norm* mosquitoes to odors from a human hand and to a synthetic attractant blend. The test population in each case comprised 75 female mosquitoes. These were transferred to the olfactometer from stock cages and allowed 1 h to adjust to the test cage environment. At the end of this time, a human subject inserted their hand into the olfactometer after which, for 3 min, the olfactometer airstream was diverted over the hand and through the test cage. The number of mosquitoes that took flight, oriented to the odor source, and were trapped in the test cage assembly was recorded. Responses were measured as percent attraction and were recorded 4 times for each mosquito strain ($n = 4$).

The same procedure was used to test mosquito responses to a synthetic attractant blend (Bernier et al. 2001) comprising 490 ml of acetone (67-64-1; 99.5+% American Chemical Society (ACS); Aldrich Chemical Company, Milwaukee, WI), 0.96 g of L-(+)-lactic acid (75-09-2; >99%; Aldrich Chemical), and 10 ml of dimethyl disulfide (624-92-0; 99%; Fluka Chemical Company, Milwaukee, WI). A 500-μl aliquot of the blend was deposited onto a 2 × 4 × 0.5-cm-wide porous plastic block made of polyethylene and polypropylene (GenPore, Reading, PA) and the block was placed into the olfactometer for 3 min. Percent attraction was measured 4 times for each mosquito strain ($n = 4$).

*Host approach, landing, and probing; and bloodfeeding time:* These tests were made to determine the average time (sec) required for each strain of mosquito to locate, land on, and commence probing on a human subject; and to complete blood engorgement on a guinea pig. In both cases, a test comprised 5 observations each for the *norm, re,* and *we* strains and was replicated 4 times ($n = 20$). The World Health Organization (WHO) insecticide resistance test apparatus (WHO 1975) was used for all tests (the test apparatus comprised two 12.5 × 4.4-cm-diameter polycarbonate chambers joined by a plastic slide gate with the opposite [open] ends of each chamber covered with 1.56-$mm^2$ mesh screen). For each test, 6 female mosquitoes were placed into the upper chamber of the test apparatus and the apparatus was secured in a vertical orientation to the forearm of a human volunteer with the screened end of the lower chamber held against the skin. The time elapsed between opening the slide gate (to allow mosquitoes in the upper chamber access to the forearm skin) and at least 1 probe of the skin by 3 of the 6 mosquitoes contained in the apparatus was used to calculate the median probe time ($PT_{50}$) (Khan et al. 1965).

To measure the average time (sec) required for each strain of mosquito to complete blood engorgement, 3 mosquitoes were placed into the WHO test apparatus and the apparatus was secured against the (shaved) abdomen of a restrained guinea pig. The elapsed time between initial insertion and complete withdrawal of the mosquito's mouthparts, for the 1st of the 3 mosquitoes in the apparatus that fed, was recorded. A single test comprised one such observation for each of the *norm, re,* and *we* strains, and was replicated 20 times ($n = 20$).

*Repellency responses:* This test was made to determine if mean repellency responses differed among the mosquito strains. Three repellents were used: 15% deet in ethanol, a commercial formulation of deet (6.65%), and a commercial formulation of citronella (10%).

To make a repellent test, two hundred 5- to 7-day-old female mosquitoes were withdrawn (Posey and Schreck 1981) from a stock cage and placed inside a 46 × 38 × 37-cm test cage. The test cage had a cotton stockinette access sleeve on the front, clear acrylic sides (for viewing), a sheet aluminum bottom, and window screen on the top and back. Sucrose solution was available to the mosquitoes at all times. Thirty minutes before commencing a test, the forearm of a human subject was treated with repellent between the elbow and the wrist at the rate of 1 ml of formulated product per approximately 650 $cm^2$ of skin surface area. A test con-

Table 1. Mean percent attraction (±SE) of *Aedes aegypti* to a human hand and to synthetic attractant in a laboratory olfactometer.[1]

| Mosquito strain | Human hand | Synthetic attractant |
|---|---|---|
| Red eye (*re*) | 12.0 (±0.75)a | 7.0 (±3.19)a |
| White eye (*we*) | 0.7 (±0.37)a | 0.3 (±0.32)a |
| Black eye (*norm*) | 54.1 (±18.35)b | 35.4 (±11.93)b |

[1] Column means followed by the same letter are not significantly different (analysis of variance, Tukey's honesty significant difference, $P > 0.05$).

Table 2. Median time, in seconds (±SE), required for *Aedes aegypti* to locate, land on, and probe human skin ($PT_{50}$) and mean time required for feeding to repletion on a guinea pig host.[1]

| Mosquito strain | $PT_{50}$ | Feeding time |
|---|---|---|
| Red eye (*re*) | 76.8 (±16.7)a | 164.7 (±10.39)a |
| White eye (*we*) | 189.6 (±20.36)b | 180.4 (±12.59)a |
| Black eye (*norm*) | 176.7 (±18.04)b | 194.1 (±13.89)a |

[1] Column means followed by the same letter are not significantly different, ($P > 0.05$).

sisted of placing the repellent-treated arm into a test cage for 3 min, at 30-min intervals (a latex glove protected the hand from bites), until the test subject received 2 or more mosquito bites in the same observation period or 1 bite each in 2 consecutive observation periods (a confirmed bite). Protection time (to the nearest one-half hour) was recorded as that elapsed between the time of repellent application and the observation period immediately preceding that in which the confirmed bite was received.

*Data analysis:* Tests of host attraction, median probe time, bloodfeeding time, and repellent activity each were made by using a completely randomized design; repellents were tested once on each of 3 human subjects. Percent attraction (after inverse sine transformation), $PT_{50}$, engorgement time, and repellency responses were analyzed by using PROC ANOVA (SAS 1988); means separation was made using Tukey's honestly significant differnce test ($P = 0.05$).

## RESULTS

### Attraction

In olfactometer tests, the mean percent attraction of *re* (12%) and *we* (0.7%) females to human hand odor was significantly different ($F = 45.47$, df = 2,9, $P \leq 0.0001$) from *norm* (54.1%) females (Table 1). Similarly, the mean percent attraction of *re* (7.0%) and *we* (0.3%) females to the synthetic attractant blend was significantly different ($F = 6.79$, df = 2,9, $P < 0.05$) from *norm* (35.4%) females (Table 1).

### Host approach, landing, and probing; and bloodfeeding time

The median time required to commence probing human skin was significantly different ($F = 12.40$, df = 2,57, $P < 0.001$) for *re* (76.8 sec) mosquitoes compared with *we* (189.6 sec) and *norm* (176.7 sec) mosquitoes (Table 2). No difference was found among mosquito strains in the mean time required to complete blood feeding (Table 2).

### Repellency responses

For each repellent, no significant difference was found in protection time attributable to mosquito strain. Differences in protection time attributable to repellent (Table 3) were significant (*re*: $F = 29.46$, df = 2,7, $P = 0.004$; *we*: $F = 62.81$, df = 2,7, $P < 0.001$; *norm*: $F = 423.47$, df = 2,7, $P < 0.0001$) and were consistent among mosquito strains; accordingly, 15% deet provided 1.5 times the protection time of 6.65% deet, whereas both provided 3–3.5 times longer protection time than 10% citronella.

## DISCUSSION

The responses of *re* and *we* females to human hand odor and to synthetic attractant in the olfactometer, when calculated as a proportion of the attraction response for *norm* females (1.00), were 0.21 and 0.01, respectively. These differences suggest the existence of a sensory, possibly visual, attraction component in *norm* females that is absent or dysfunctional in *re* and *we* mosquitoes. The lack of ommatidial screening pigments in eye-color mu-

Table 3. Mean protection time, in hours (±SE), from bites by *Aedes aegypti* when 3 different mosquito repellents.[1]

| | Repellent | | |
|---|---|---|---|
| Mosquito strain | 15% deet[2] | 6.65% deet | 10% citronella |
| Red eye (*re*) | 4.5 (±1.0)a/A | 3.0 (±0.5)a/B | 1.0 (±0.0)a/C |
| White eye (*we*) | 5.5 (±1.0)a/A | 3.5 (±0.5)a/B | 1.0 (±0.5)a/C |
| Black eye (*norm*) | 5.5 (±0.5)a/A | 3.5 (±0.5)a/B | 1.0 (±0.5)a/C |

[1] Column means followed by the same lowercase letter are not significantly different (analysis of variance, Tukey's honestly significant difference, $P > 0.05$). Row means followed by the same uppercase letter are not significantly different (analysis of variance, Tukey's honestly significant difference, $P > 0.05$).

[2] deet, diethyltoluamide.

tants (Bhalla 1968a, 1968b; McDonald and Rai 1970) seems an obvious possibility (these affect visual acuity and the perception of movement [Clements 1999]), but we have no basis for inferring that the genes responsible for eye color also condition host preference or attraction. Yet other factors may have affected attraction responses for *Ae. aegypti,* including biases induced by the testing (olfactometer) apparatus. Despite the absence of a visible human stimulus, for example, the responses of each mosquito strain to synthetic attractant (when calculated as a proportion of the total response for all 3 strains) were of approximately the same ratio as those to the hand (synthetic attractant/human hand: *re*: 0.164/0.180; *we*: 0.007/0.01; *norm*: 0.829/0.810). But because the olfactometer restricts vision and flight somewhat, females could have found the host source in our tests by odor, heat, or by crawling toward it. Thus, it may have been possible to obtain a different set of responses from an assay system that clearly allows females to use vision to fly to the host.

The *re* females required the least amount of time to locate, land on, and probe for feeding sites on a human subject. Given the technique used to measure these responses, it is apparent that stimuli other than (or in addition to) those eliciting attraction in the olfactometer influence host approach and landing. The WHO apparatus functions without forced air flow (Feinsod and Spielman 1979), thus mosquitoes placed in the top chamber respond to stimuli in the convection currents that rise from the skin or to the convection currents themselves (Khan et al. 1966, 1967). Alighting on the skin is stimulated by sweat volatiles other than lactic acid (water vapor and $CO_2$ provide supplementary stimuli), whereas the presence of moisture enhances probing of the skin surface (Clements 1999). In any case, our results show that when probing time responses are measured with *re* females, estimates of $PT_{50}$ will be shorter than for *we* and *norm* females. This fact would call into question any data from studies that rely upon landing rate responses of *re* females or that seek to correlate landing rates of *re* females with the numbers of *re, we,* or *norm* females collected by other means. Repellent test results support this hypothesis because comparatively short protection times were observed for deet-based repellents against *re* females. These mosquitoes locate, land on, and probe human skin in 60% less time than *we* and *norm* females. The resulting increased biting pressure can expedite repellent failure (Barnard et al. 1998).

We noted earlier that our study was made to examine the hypothesis that selected fitness parameters for normal and genetic marker strains of mosquitoes were no different. We failed to accept this hypothesis in 3 of the 4 cases tested. In fact, we showed that some mosquito strains with marker genes for eye color were not attracted to human hosts, or to synthetic attractant, at the same rate as mosquitoes with normal eye color and that host location, landing, and probing of the skin occurred more quickly in one mutant strain (*re*) than in other mosquitoes. We also found that *re* females, on average, failed to respond to deet-based repellents on human skin in 15% less time than *we* and *norm* strains.

These inferences of fitness are based on laboratory observations of genetic strains of *Ae. aegypti*; this study was not an analysis of gene flow. Nevertheless, our results lend support to a growing concern (Scott et al. 2002) that conventional knowledge of mosquito natural history is inadequate for the successful implementation of control technologies that seek to use genetically aberrant or modified mosquitoes. We believe this concern may be justified given that, in most cases, we have yet to obtain relevant comparative data for counterpart mosquito populations in nature. Perhaps the most important concern in this regard is that manipulation of genetic (and possibly other) factors in the mosquito population has the potential to result in assortative behavior and subdivision of mosquito populations. Differential regulation of subpopulations could lead to an advantage for one over the other (Scott et al. 2002) and the emergence of untoward effects or the failure of parasite and vector control strategies.

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